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Differential effects of human SP-A1 and SP-A2 variants on phospholipid monolayers containing surfactant protein B

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Abstract

Surfactant protein A (SP-A), the most abundant protein in the lung alveolar surface, has multiple activities, including surfactant-related functions. SP-A is required for the formation of tubular myelin and the lung surface film. The human SP-A locus consists of two functional SP-A genes, *SP-A1* and *SP-A2*, with a number of alleles characterized for each gene. We have found that the human in vitro expressed variants, SP-A1 ($6A^2$) and SP-A2 ($1A^0$), and the coexpressed SP-A1/SP-A2 ($6A^2/1A^0$) protein have a differential influence on the organization of phospholipid monolayers containing surfactant protein B (SP-B). Lipid films containing SP-B and SP-A2 ($1A^0$) showed surface features similar to those observed in lipid films with SP-B and native human SP-A. Fluorescence images revealed the presence of characteristic fluorescent probe-excluding clusters coexisting with the traditional lipid liquid-expanded and liquid-condensed phase. Images of the films containing SP-B and SP-A1 ($6A^2$) showed different distribution of the proteins. The morphology of lipid films containing SP-B and the coexpressed SP-A1/SP-A2 ($6A^2/1A^0$) combined features of the individual films containing the SP-A1 or SP-A2 variant. The results indicate that human SP-A1 and SP-A2 variants exhibit differential effects on characteristics of phospholipid monolayers containing SP-B. This may differentially impact surface film activity.

Keywords: Genetic variant; Interaction of surfactant protein and lipid; Organization of phospholipid monolayer; Surfactant protien A (SP-A); Surfactant protien B (SP-B)

1. Introduction

Pulmonary surfactant is essential for normal lung function. Structurally, it is dynamic and a number of morphologic forms have been characterized for surfactant. These, among others, include lamellar bodies found both intracellularly and extracellularly, and tubular myelin and the surface film found extracellularly. Its composition is about 90% lipids and 10% proteins. The surfactant-associated proteins (SPs) include SP-A, SP-B, SP-C, and SP-D. The primary lipid is dipalmitoylphosphatidylcholine (DPPC), which comprises over 40% of the material. Other lipids present in relatively substantial amounts are unsaturated phosphatidylcholine (PC), phosphatidylglycerol (PG), and cholesterol [1]. SP-A, an asialoglycoprotein of complex architecture, plays an important role in the structure, metabolism, and the surface-tension lowering activity of surfactant [2–7], host defense [8–10], and in parturition, serving as a hormone [11].

The human SP-A locus consists of two functional genes, *SP-A1* and *SP-A2*, in opposite transcriptional orientation, and a pseudogene between the *SP-A1* and *SP-A2* genes [12]. Both, the cDNAs and the genomic sequences have been cloned and characterized [13–15]. Based on nucleotide differences within the coding region, more than 30 alleles (or variants) for both genes have been characterized in part or fully [16,17]. Ten of

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-3-phosphocholine; CHO, Chinese Hamster Ovary (CHO)-K1 cell line; LE phase, liquid-expanded phase; LC phase, liquid-condensed phase; PG, L-α-phosphatidylglycerol (from egg PC, sodium salt); SP-A, surfactant protein A; SP-B, surfactant protein B

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these variants are found in the general population at a frequency of 0.01. Four of these are for SP-A1 (i.e. 6A, $6A^2$, $6A^3$, $6A^4$), and six are for SP-A2 (i.e. 1A, $1A^0$, $1A^1$, $1A^2$, $1A^3$, $1A^5$). Some of the nucleotide polymorphisms result in amino acid substitutions [18], and collectively SP-A1 and SP-A2 variants differ at 10 amino acid residues, including a cysteine at position 85. Native SP-A is an octadecamer that consists of six trimers, and it has been suggested that each trimer consists of two SP-A1 and one SP-A2 gene products [19]. However, the mRNA ratio of SP-A1 and SP-A2 differs from the 2:1 ratio proposed for protein structure [20], indicating that single gene products may exist in homo-oligomeric structures. Moreover, recent evidence indicates that the relative amounts of SP-A1 and SP-A2 gene products differ as a function of lung health status [21].

In vitro expressed single gene products of human SP-A1 or SP-A2 have been shown to be functional, and with differences in biochemical and biophysical properties, as well as in other functions [22–26]. Previous findings on the expressed protein products of the SP-A1 and SP-A2 alleles [24] have indicated that the two gene products exhibit the following differences: in oligomerization of the protein under appropriate conditions; in the structural stability of their collagen domains, with the SP-A2 products being more stable than those of SP-A1; in ability to cause aggregation of lipopolysaccharides and liposomal lipids, with SP-A2 products being more potent in causing aggregation than SP-A1 products. In addition, a number of SP-A variants have been shown to associate with several pulmonary diseases including respiratory distress syndrome in the prematurely born infant [22,27–31]. A synergistic effect between SP-A and SP-B variants in RDS susceptibility has also been observed [31–34].

SP-A and SP-B are necessary for the formation of tubular myelin [5,6], and SP-A has been shown, in vitro, to have an impact on the regional organization of phospholipid monolayers containing SP-B or SP-C [35,36]. The question addressed in this report is whether the two human SP-A1 and SP-A2 gene products have a differential influence in the organization of phospholipid monolayers, especially monolayers containing phospholipids and the surfactant protein SP-B [36]. To explore this, we studied the interaction of human in vitro expressed SP-A variants (i.e. the $6A^2$ of SP-A1, the $1A^0$ of SP-A2, and the co-expressed $6A^2/1A^0$ product of SP-A1/SP-A2) with SP-B, in phospholipid monolayers. The SP-A variants used in the study were expressed *in vitro* from stably transfected mammalian Chinese Hamster Ovary (CHO) cells. The results indicated differences between the two SP-A variants in their interaction with SP-B.

2. Materials and methods

2.1. Experimental materials

DPPC and egg PG were obtained from Avanti Polar Lipids (Birmingham, AL), and were used as received after verification of their purity by thin layer chromatography. Water was deionized and doubly distilled, and the second distillation being from dilute potassium permanganate solution. Other chemicals were obtained from Fisher Scientific Co. (Ottawa, ON, Canada) or Sigma (St. Louis, MO).

2.2. Human SP-A1 and SP-A2 variants from in vitro expression CHO cells

The mammalian Chinese Hamster Ovary (CHO)-K1 cell line (American Type Culture Collection, Manassas, VA, Cat. CCL 61) was used as the host to express human SP-A variants. The cell culture techniques and the culture media were previously described [37]. Stably transfected CHO cell lines that expressed human SP-A1 ($6A^2$) or SP-A2 ($1A^0$) were obtained through transfection and selection, as described previously [37]. To express SP-A variants, cells were grown to confluence in the growth medium with fetal bovine serum, then the growth medium was removed and expression medium, which did not contain fetal bovine serum but had 0.5 mM ascorbic acid, and 40 mg of proline per liter medium was added. The medium containing secreted SP-A protein was harvested after 5 days in culture, and SP-A variants were recovered and purified from the culture medium using mannose-affinity chromatography, as described previously [26]. Purified SP-A was concentrated using Amicon Centriprep-10 concentrators (Amicon, Beverly, MD).

2.3. Native human SP-A from BAL fluid

The native human SP-A was purified from BAL fluid obtained from alveolar proteinosis patients using a butanol-extraction method as described [38,39] with slight modification. In brief, after complete extraction of whole BAL surfactant with butanol, the mixture was centrifuged at 5000×*g* at 15 °C for 30 min and the supernatant, which contains the butanol, was discarded. The pellet was then completely dried with a flux of nitrogen gas. The dry pellet was homogenized in 24 ml of the OBG buffer (20 mM n-Octyl β-D-Glucopyranoside, 10 mM HEPES, 150 mM NaCl, pH 7.4) and centrifuged at 210,000×*g* at 15 °C for 30 min, and then the supernatant containing detergent-soluble proteins and potential trace amount of butanol was discarded. The above procedure was repeated twice. After a final pelleting, the detergent-insoluble protein was dissolved in 5 ml of buffer (5 mM Tris/HCl, pH 7.4) and dialyzed extensively for 48 h against 4 l of the same buffer with four changes of buffer. The dialyzed solution was centrifuged at 155,000×*g* at 4 °C for 30 min and the supernatant containing SP-A was collected and kept at −80 °C.

With regards to the potential surface activity of butanol that was used early in the purification procedure, we have always been particularly cautious about avoiding any effects of residual surface active material by diluting and dialyzing our samples quite extensively. In this case, the butanol used in the early step was removed immediately by centrifugation and pellet dryness procedure. The pellet was then resuspended in 24 ml OBG buffer (see above) and centrifuged to remove any contaminating trace of butanol (this procedure was repeated 3 times). The final pellet was dissolved in 5 ml of buffer (see above) and dialysed against 0.8×10^3 fold buffer with four changes of the buffer. Therefore, the dilution factor is estimated to be about 10^{15}. We have never observed abnormal effects that we could attributed to butanol or other materials in our studies with SP-A.

All procedures were performed at 4 °C or on ice. Protein concentration of SP-A was determined using the Micro-BCA method of Smith et al. [40] (Pierce, Rockford, IL) with RNase A as a standard. SP-A was aliquoted and stored at −80 °C, until use.

2.4. Porcine SP-A and porcine SP-B preparation

Porcine SP-A and SP-B were prepared as described before [41]. In brief, pig lungs were lavaged with 150 mM NaCl, and the lavage was centrifuged at 800×*g* for 10 min. The pellet was removed and the supernatant was centrifuged again at 7000×*g* for 60 min. The pellet was used for isolation of either SP-A or SP-B. SP-A was obtained from the pellet by extraction with 1-butanol and further purified as described above [38,42]. SP-B was prepared from the pellet using chloroform–methanol as described previously [43,44].

2.5. Monolayer epifluorescence analysis

Monolayer fluorescence measurements were performed in the following way. DPPC or DPPC:eggPG (8:2, mol/mol) and SP-B were mixed in chloroform:

methanol and 1 mol% of NBD-PC (based on phospholipid content) was added. Monolayers were formed by spreading of the lipid–protein mixture containing 17 wt.% SP-B on subphases of 145 mM NaCl, 5 mM Tris, 5 mM $CaCl_2$ (pH 6.9) in the presence of SP-A (subphase concentration 0.68 μg/ml). The apparatus used in these experiments has been described previously [45]. A modified monolayer trough was employed to reduce the subphase volume and surface area by about 10-fold from that of the original trough in order to study the small amounts of gene products that were available at subphase concentrations appropriate to those employed in previous studies of SP-A in monolayers systems [35,36]. Experiments were performed in a small Teflon trough with a volume of 12.5 ml [36], at a temperature of 21–23 °C. The initial spreading surface pressure was about 5 mN/m. After spreading, 1 h was allowed for adsorption of SP-A to the spread monolayers during which time the subphase was stirred continuously. To increase the surface pressure of the films small aliquots of SP-B/DPPC were added to the surface and 5 min were allowed for evaporation of solvent and for equilibration of the films. At each surface pressure the monolayers were observed through the fluorescence of NBD-PC which partitions into the liquid-expanded (LE) phase of the lipid monolayer [36,46]. Each experiment was repeated at least twice.

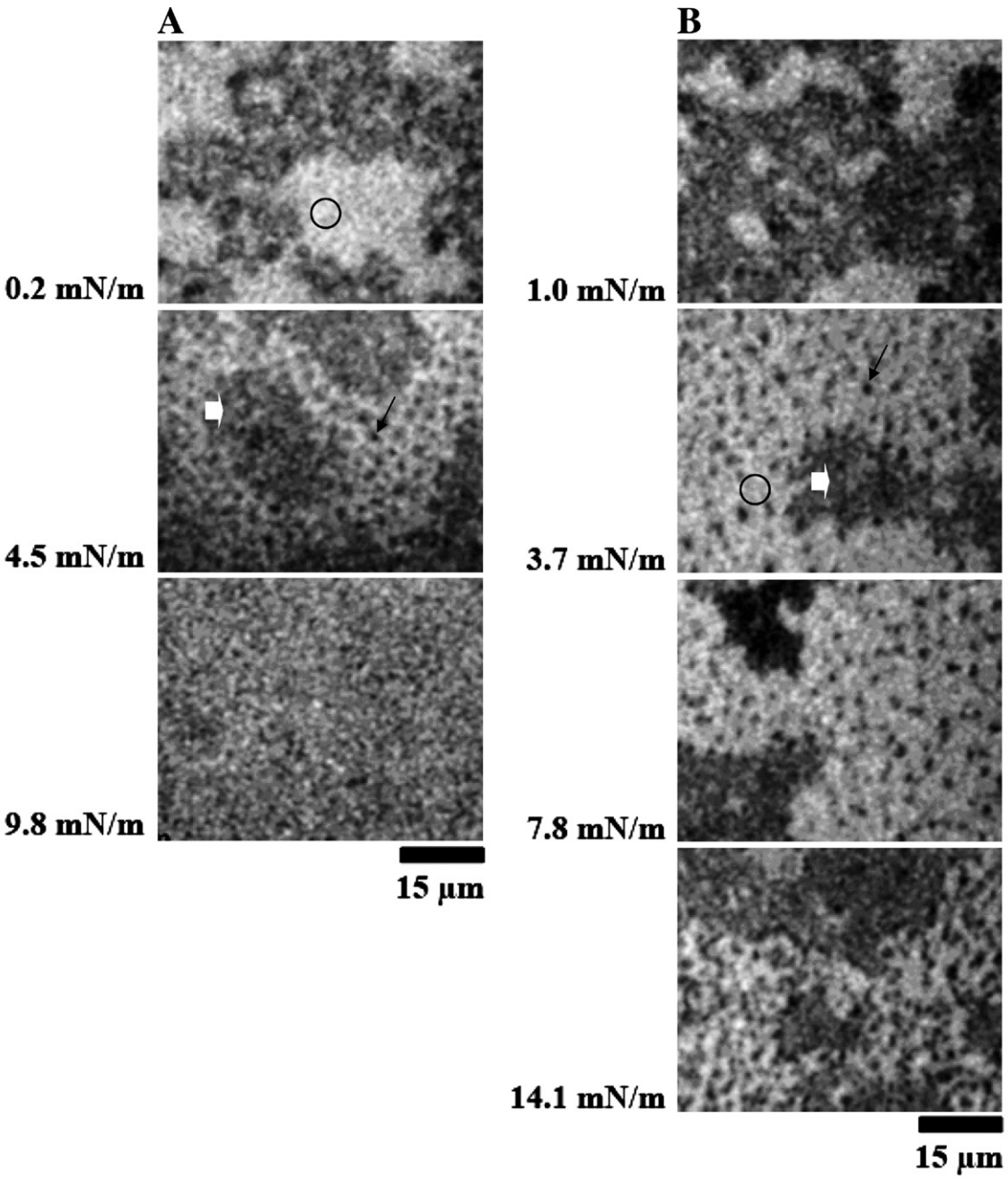

Fig. 1. Image of monolayers of DPPC containing 17% SP-B spread over a subphase of 0.68 μg/ml of porcine SP-A (panel A) and human SP-A (panel B). Surface pressures at which images were obtained are given at the sides of the figure. Three phases in the images were observed, including lipid liquid expanded (LE) phase (bright regions, circles), lipid liquid-condensed (LC) phase (small black domains, arrows), and surface clusters characteristic of SP-A and SP-B complexes (grey regions, block arrows). The morphology of the DPPC monolayers containing SP-B and human SP-A in panel B was similar to that of the lipid monolayers plus SP-B and porcine SP-A in panel A.

3. Results

3.1. Effect of porcine SP-A in DPPC monolayers with porcine SP-B

Fig. 1, panel A shows epifluorescence images at different surface pressures of monolayers of DPPC and porcine SP-B spread on subphases containing porcine SP-A. The images show the presence of three phases: lipid liquid expanded (LE) phase (bright regions, circles), lipid liquid-condensed (LC) phase (small black domains, arrows), and surface clusters characteristic of SP-A/SP-B complexes (grey regions, block arrows). In similar experiments with SP-A and SP-B labeled with two different fluorophores we have shown that the surface clusters, seen as grey phase in the images, are specific for the combination of SP-A and SP-B, and they are comprised of the two proteins and possibly some lipids [36].

These control studies showed that the different methodology used to form and compress the monolayer that was used in these experiments gave results which were consistent with monolayers formed on a larger trough and compressed mechanically using a ribbon barrier, the more "conventional" way to carry out monolayer compression.

3.2. Effect of human SP-A in DPPC or DPPC:PG monolayers with porcine SP-B

Fig. 1B shows images at different surface pressures of monolayers of DPPC/SP-B spread on solutions of human SP-A. A surface phase (grey clusters in the images, block arrows), in addition to the conventional lipid LE and LC phases, is detectable. The morphology of the DPPC monolayers containing SP-B and human SP-A was similar to that of the lipid monolayers plus SP-B and porcine SP-A (Fig. 1A). These experiments showed that human SP-A and porcine SP-B formed the same type of protein-rich complexes as were seen with porcine SP-A in previous studies [36].

Many of the previous studies on the SP-A and SP-B interactions in monolayer experimental systems had been carried out in the presence of DPPC plus 20% PG [36]. The influence of PG on the interactions of human SP-A and porcine SP-B was therefore investigated. Fig. 2 represents images of monolayers of DPPC:eggPG (8:2, mol/mol) plus SP-B spread on human SP-A. The data showed that the interactions between SP-A and SP-B produced the protein-rich phase (grey clusters, block arrows) in the monolayers regardless of the presence of 20 mol% unsaturated PG.

3.3. Effect of SP-A1 variant ($6A^2$) in DPPC monolayers with porcine SP-B

Fig. 3A and B shows images at different surface pressures of two experiments with monolayers of SP-B/DPPC spread on subphases containing SP-A1 variant ($6A^2$). The images reveal three characteristic features typical for the monolayers: a fine network of grey phase (block arrows), which extended throughout the LE phase, large protein-rich patches (large dark grey regions, stars), and LC phase (small black domains, arrows). The SP-A1 molecules were likely localized in the network in the lipid LE phase and in the large characteristic patches seen in the monolayers. The distribution of the grey network throughout the LE phase was different than any of the images seen before with SP-A and SP-B in any of these monolayer systems, suggesting that the $6A^2$ variant of SP-A1 had a different type or extent of interaction with SP-B, and possibly lipids, than did "native" SP-A or the allele $1A^0$ of SP-A2 protein (see below).

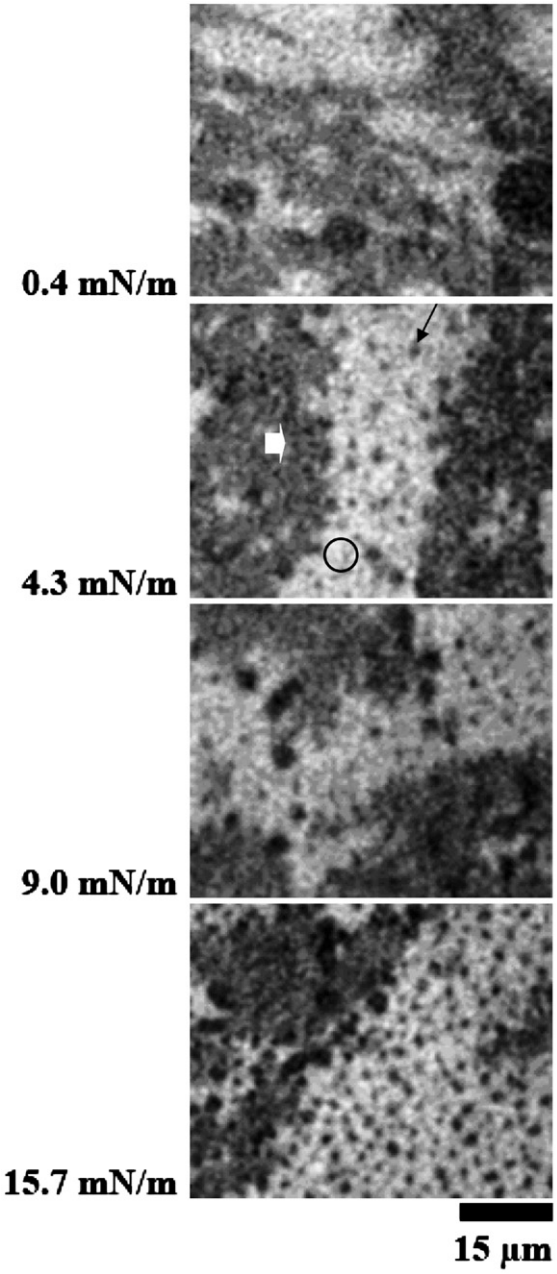

Fig. 2. Images of films of DPPC: egg PG (8:2, mol/mol) containing 17% SP-B spread over human SP-A at 0.68 μg/ml. Surface pressures are given at the side of each image. In these images of monolayers of DPPC:eggPG plus SP-B spread on human SP-A show that the interactions between SP-A and SP-B produced the protein-rich phase (grey clusters, block arrows) in the monolayers regardless of the presence of 20 mol% unsaturated PG.

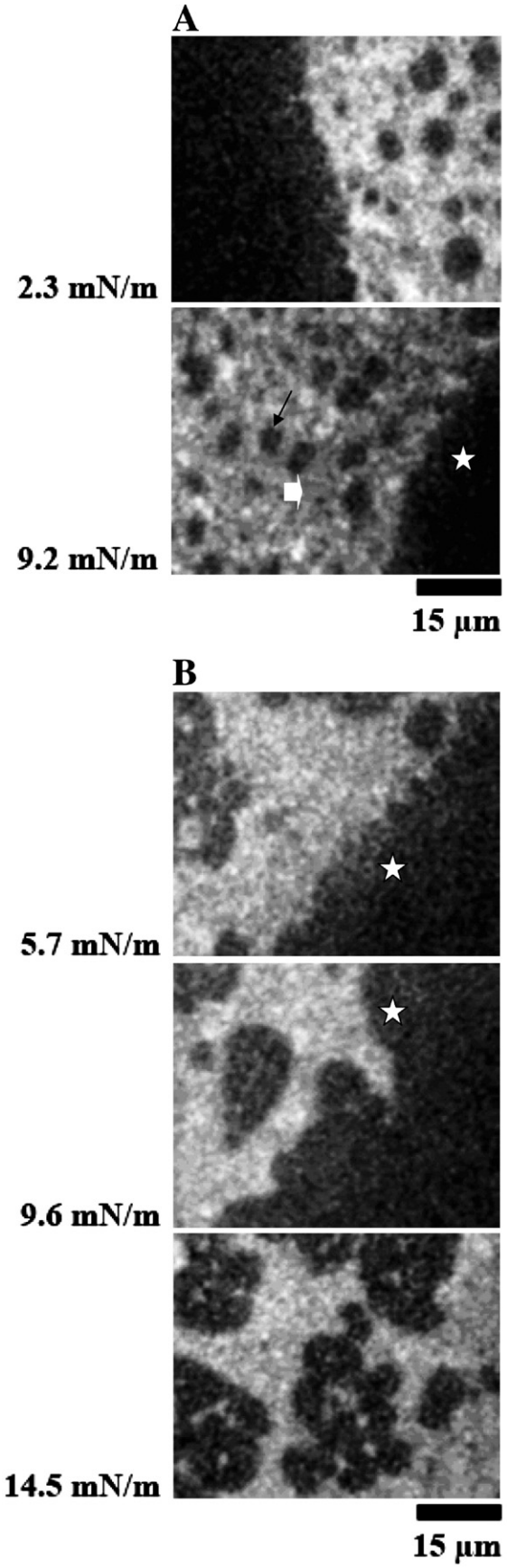

Fig. 3. Images from films of DPPC with SP-B (17%) spread over the product of the $6A^2$ of SP-A1 (0.68 μg/ml). Surface pressures are given at the side of each image. Panels A and B are from separate experiments. Both panels A and B show three characteristic features typical for the monolayers: a fine network of grey phase (block arrows) which extended throughout the LE phase, large protein-rich patches (large dark grey regions, stars), and LC phase (small black domains, arrows).

3.4. Effect of SP-A2 variant ($1A^0$) in DPPC monolayers with porcine SP-B

Fig. 4A and B shows results at different surface pressures from two experiments with monolayers of SP-B/DPPC spread on subphases containing the protein product of allele $1A^0$ of SP-A2. The morphology of the films was very similar to that observed in the films containing combinations of SP-B and porcine SP-A (Fig. 1A) or human SP-A (Figs. 1B and 2). In this case, the probe-excluding phase (block arrows), which was characteristic for the phospholipid monolayers containing combinations of SP-B and native SP-A, co-existed with, but was separate from, the lipid LE (bright regions, circles) and LC (black domains, arrows) phases.

3.5. Effect of the coexpressed SP-A1/SP-A2 ($6A^2/1A^0$) protein in DPPC monolayers with porcine SP-B

Fig. 5A and B represents images at different surface pressures from two experiments with monolayers of SP-B/DPPC spread on subphases containing the coexpressed SP-A1/SP-A2 ($6A^2/1A^0$) protein. The morphology of the films containing the coexpressed SP-A1/SP-A2 ($6A^2/1A^0$) protein appears to combine features of the lipid–protein monolayers containing SP-A1 (Fig. 3) and SP-A2 (Fig. 4). Large likely protein-rich patches (stars), similar to those seen in the monolayers spread on SP-A1 (Fig. 3) and a network of grey phase (block arrows) extended throughout the LE phase in the films containing the coexpressed SP-A1/SP-A2 ($6A^2/1A^0$) protein. In some cases, however, the grey phase appeared to segregate from the LE phase and regions of the traditional lipid LE phase were seen in the films (bright regions (circle) in Fig. 5B). In this property the morphology of the system resembled that of the films of SP-B and DPPC spread on SP-A2 (Fig. 4).

4. Discussion

Recent work has provided evidence for specific interaction of the porcine proteins SP-A and SP-B in monolayers of lipids [35,36]. The ability of monolayers containing SP-B to attract SP-A into the surface were much greater than those containing SP-C where little or no interaction with SP-A occurred [36,41]. The interaction between SP-B and SP-A was dependent on the presence of lipid, especially phosphatidylcholine [36]. In this paper, we investigated interactions between SP-B and in vitro expressed SP-A1 and SP-A2 gene variants in the formation of lipid monolayers in the presence of DPPC. The results, (a) confirmed the interactions between SP-B and human SP-A from alveolar proteinosis fluid in monolayers containing DPPC with or without the presence of unsaturated PG; (b) showed

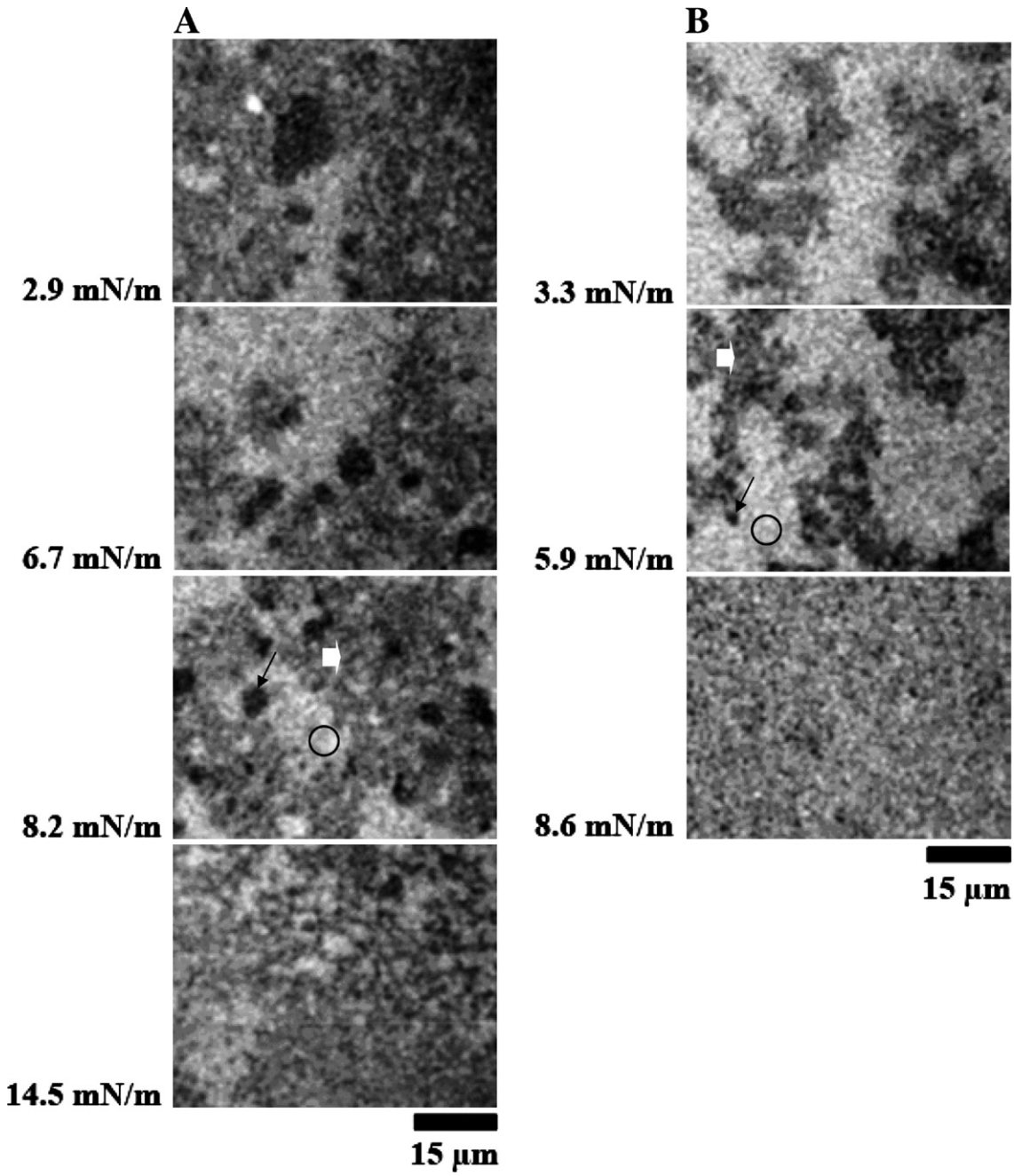

Fig. 4. Images from films of DPPC/SP-B (17%) spread over the product of the $1A^0$ of SP-A2 (0.68 μg/ml). Surface pressures are given at the side of each image. Panels A and B are from separate experiments. The morphology of the films was very similar to that observed in the films containing combinations of SP-B and porcine SP-A (Fig. 1A) or human SP-A (Fig. 1B).

differences in the interaction of the protein products of each of the two human SP-A genes. These differences were manifested in the organizational states in the monolayer in which the SP-A and SP-B complex is found; (c) the $1A^0$ variant of SP-A2 formed complexes similar to those seen before, with porcine SP-A or with native human SP-A from alveolar proteinosis fluid; (d) while the $6A^2$ variant from SP-A1 also formed a surface complex, its organization and distribution differed from that formed by native SP-As or by the $1A^0$ variant. In the case of $6A^2$, the complex observed was distributed in large patches and an extensive network rather than in clusters in the LE phase as they were when the $1A^0$ or native SP-A was present; (e) coexpressed $1A^0/6A^2$ SP-A showed a surface complex formation with characteristics of both complexes (i.e. when the $1A^0$ or $6A^2$ protein was used alone). These observations indicate a differential impact of SP-A1 and SP-A2 products in film formation and film regional organization including the organization of film lipid-protein complexes that may influence overall surface activity and other characteristics of the surface in the lung.

The network formed, when the $6A^2$ product was present, appeared to extend throughout the region of LE phase (that is

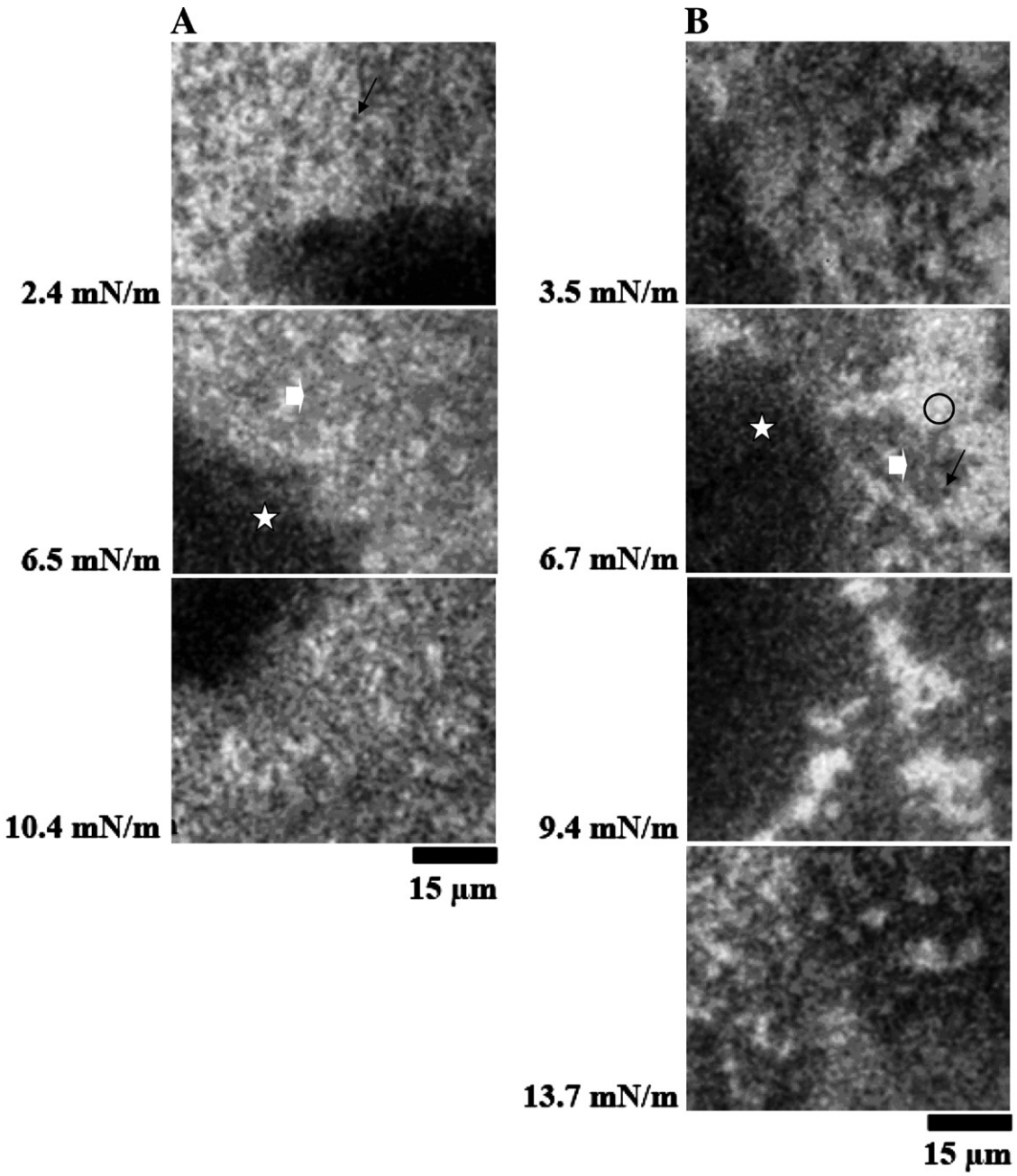

Fig. 5. Images of films of DPPC/SP-B (17%) spread over a coexpressed product ($1A^0/6A^2$) of SP-A1 and SP-A2 (0.68 μg/ml). Surface pressures are given at the side of each image. Panels A and B are from different experiments. The morphology of the films containing SP-A1 and SP-A2 variants appears to combine features of the lipid–protein monolayers containing SP-A1 (Fig. 3) and SP-A2 (Fig. 4). Large likely protein-rich patches (stars), similar to those seen in the monolayers spread on SP-A1 (Fig. 3), and a network of grey phase (block arrows), similar to those seen in the monolayer spread on SP-A2 (Fig. 4), are observed in the films containing SP-A1 and SP-A2.

the fluid or mobile phase) of the lipid. It is unlikely that the material was "dissolved" in the LE phase, but that the LE phase provided a convenient (fluid) environment for the material to form and lodge. While previous studies have shown that for porcine SP-A the clustered complex formed contained both SP-A and SP-B [36], this has not been shown definitively for human SP-A and the SP-A variants. Nevertheless, it seems to be a very reasonable assumption that all the grey phase complexes seen in the monolayers described here contain both SP-A and SP-B. One cannot obtain a reliable estimate of the extent of the phases with the current technology. Therefore, one cannot tell if the extent or strength of interaction of SP-B with one allelic product is different than with the other. Nor can one tell the stoichiometry of the complexes. There is, however, a remarkable difference observed in the shapes and locations of the complexes in the monolayer.

Previous studies indicate that the two SP-A variants ($1A^0$ and $6A^2$) differ in six amino acid residues within the coding region

[16–18,47]. These amino acid substitutions, based on the SP-A precursor numbering are shown in the Table.

Amino acid no.	19	66	73	81	85	91
$1A^0$	Ala	Thr	Asn	Val	Arg	Ala
$6A^2$	Val	Met	Asp	lle	Cys	Pro

Recent work has shown that removal of the signal peptide results in molecules with various N-terminal sequences [26]. Some molecules may and others may not contain amino acid 19. However, in either case, alanine and valine are relatively small molecules and represent conservative amino acid differences, and therefore it is unlikely that changes in amino acid 19 have a major impact on SP-A function. The remaining amino acid changes are located within the collagen-like domain of SP-A and, as such, may play a role in the structural stability of SP-A [24], or other functions [48–50].

We have shown previously that SP-A1 has a lower thermal stability than SP-A2 [24] and suggested that this difference is due to differences in amino acid 85. A cysteine at residue 85 may affect stability in the triple helix resulting in local micro-unfolding. Studies on the stability of the tripeptide unit $(Gly–X–Y)_n$ with different substitutions at the X and Y positions have shown that the collagen triple-helix with guest triplets of Gly–Glu–Arg had a higher stability than 40 other different guest triplets tested [51,52]. We have also shown that in-vitro expressed SP-A1 is identified with higher size oligomers than SP-A2 [26], and speculated that amino acid 85 contributes to oligomerization differences. Whether SP-A1 and SP-A2 differences, in the structural stability, oligomerization, or both, contribute to the film differences observed in this study, is currently unknown. However, recent findings from our laboratory indicate that amino acid 85 plays indeed an important role in characteristics that distinguish SP-A1 and SP-A2 [53].

Moreover, differences in other biophysical and biochemical properties have been observed between SP-A1 and SP-A2 [22,24,26]. These include self-aggregation, phospholipid and lipopolysaccharide aggregation, differences in aggregation and oligomerization pattern in response to ozone oxidation, and others. The results described here extend the range of differences between SP-A1 and SP-A2 products observed previously in solution [24] to the influence of the SP-A1 and SP-A2 variants on lipid monolayers containing SP-B. The lipid–protein rearrangements caused by SP-A1 and SP-A2 seen in this work are consistent with the influence of these products on aggregation [24] in that the SP-A2 product ($1A^0$) had a greater influence on the monolayers than did the SP-A1 product ($6A^2$). Also, as in the previous observations in solution [24] the SP-A2 product behaved more like native SP-A in lipid–protein monolayers than did the SP-A1 product. Functional differences between SP-A1 and SP-A2 in proinflammatory cytokine production [25,54], inhibition of surfactant secretion [26], and ability of SP-A variants to enhance phagocytosis [23,55] have also been observed. These findings, along with the present observations indicate that the properties and function of the SP-A1 ($6A^2$) and SP-A2 ($1A^0$) differ, and the mechanisms through which these contribute to diseases such as RDS [28,30–33] are likely to differ. However, we speculate that a normally functioning lung may depend on the relative levels of SP-A1 and SP-A2 and not the total levels of SP-A. Support for this postulate is provided by our recent findings where the ratio of SP-A1 to total SP-A differs as a function of lung health status [21].

In summary, this work indicates that differences in the nature of physical interaction between SP-A variants with SP-B exist. We speculate that these differences account for differences in physiological properties of the surface monolayer, and therefore an appropriate SP-A1 to SP-A2 content may be critical for lung health. Additional work is necessary to understand more fully the precise nature of these differences in physical properties and their consequences for surfactant function.

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